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19 ABSTRACT (Continue on reverse if necessary and identify by block number)

The primary objectives of this research are to investigate pressure effects on extreme thermophiles isolated from submarine hot vents, to compare the effects of pressure on deep sea and shallow-water organisms, and to determine the properties of hydrogenases as a function of temperature and pressure. During the first year we investigated the importance of interphase mass transfer on the growth of Mc.jannaschii at elevated pressures and determined conditions under which growth is transport limited. The results suggest that transport limitations may govern the growth rate of the organism in its natural deep-sea habitat. We also demonstrated that the combination of high pressure and high NaCl concentration has a potent stabilizing effect on crude hydrogenase from Mc. jannaschii. The interplay between pressure and salt may also be important in the deep-sea environment. In the second year, further studies of bacterial growth and productivity at elevated pressures will proceed in parallel with studies of hydrogenase structure and function. The organisms to be studied include Mc.jannaschii, Mc.thermolithotrophicus, and the newly

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ANNUAL PROGRESS REPORT

March 26, 1990

CONTRACT NUMBER N00014-89-J-1884 R&T CODE 441d024

PRINCIPAL INVESTIGATOR: Douglas S. Clark

CONTRACTOR: University of California, Berkeley

CONTRACT TITLE: Pressure-Temperature Effects on Thermophilic Archaebacteria

START DATE: 1 March 1989

PERIOD OF PERFORMANCE: 1 March 1989 - 28 February 1990

RESEARCH OBJECTIVES: To investigate pressure effects on archaebacteria by examining pressure-temperature relationships in the behavior of extreme thermophiles isolated from submarine hot vents; to compare the effects of pressure on deep-sea and shallow-water organisms [e.g, the methanogens *Methanococcus jannaschii* and *Methanococcus maripaludis*, and the newly isolated extremely thermophilic archaebacterium ES4 (Pledger and Baross, Appl. Environ. Microbiol., in press)] under conditions that approximate the known physicochemical environments of deep-sea hydrothermal vents; to determine the properties of hydrogenases isolated from thermophilic methanogens as a function of temperature and pressure.

PROGRESS (Year 1)

Improvements in the High Pressure-Temperature Bioreactor: The larger of our two high pressure-temperature reactors has recently been equipped to enable fully automatic sampling of gas samples from the reactor vessel. Specifically, three pneumatic valves were incorporated into the gas outlet of the reactor. The valves can be actuated manually by switching or automatically by the gas chromatograph via a sample-event controller. Automatic sampling will serve two purposes: the sample volume will be more reproducible, and sampling will be possible around-the-clock without human supervision. During sampling, a hard copy of the chromatogram is produced and the data are automatically down-loaded to the IBM AT hard drive for further analysis.

Pressure-Effects on Methanococcus jannaschii: We have recently investigated the importance of inter-phase mass transfer on the growth of *M. jannaschii* at elevated pressures. Previous experiments in our laboratory demonstrated that the growth rate of *M. jannaschii* (as measured by protein and methane production) at both 86°C and 90°C increased significantly with pressure up to 750 atm. These studies were performed in a high temperature-pressure reactor with continuous recirculation of gaseous substrate through the liquid phase. However, when cultures were incubated under quiescent conditions (no gas recirculation), methane production was linear and the growth rate was unaffected by pressure (Figure 1). These results indicate that growth without gas circulation is limited by the rate of interphase mass transfer. Transport limitations may also govern the growth rate of the organism in its natural deep-sea habitat. For example, if the supply of gaseous substrate is relatively low *in situ*, it is possible that *M. jannaschii* naturally behaves as a barotolerant rather than a barophilic organism.

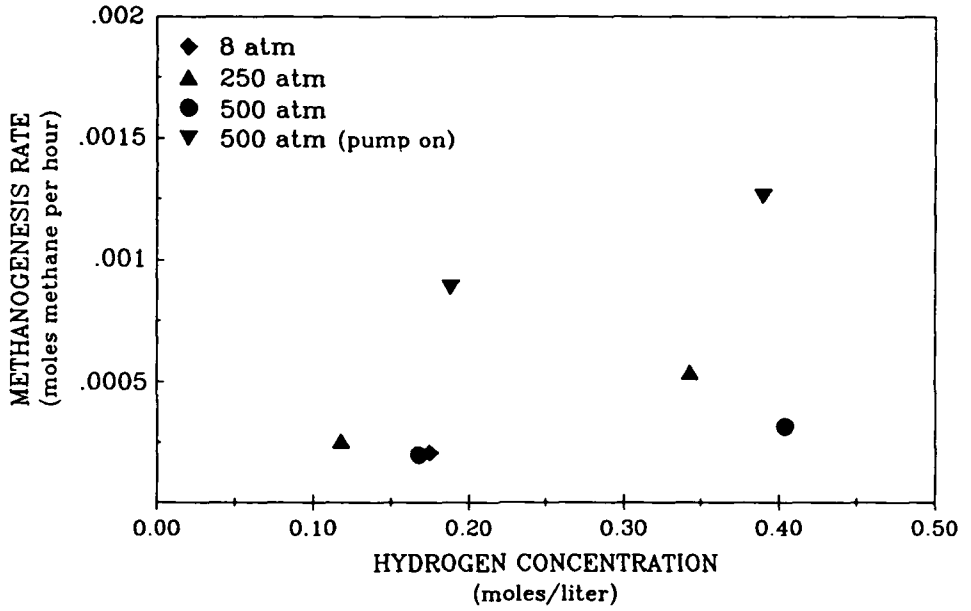

Figure 1. Growth rates of *M. jannaschii* as a function of pressure with (pump on) and without gas recirculation through the reactor vessel. Methanogenesis rates with the pump on were estimated from methane produced during the first three hours of growth. "Hydrogen concentration" refers to the average hydrogen concentration during the growth experiment.

Growth of the extremely thermophilic archaebacterium ES4: We also plan to examine the effects of pressure on the recently isolated deep-sea vent organism ES4 (Pledger and Baross, Appl. Environ. Microbiol., in press). To date, we have grown ES4 at ~ 1 atm, pH 6.0, up to 105°C in the artificial seawater medium described by Brown and Kelly [Appl. Environ. Microbiol., 55, 2086 (1989)]. Growth studies as a function of pressure will soon commence.

Pressure-Induced Stabilization of Hydrogenase: The thermal stability of hydrogenase(s) from *M. jannaschii* is being studied as a function of pressure. Interestingly, enzyme stability at high and low pressure is dramatically affected by the presence of NaCl. As shown in Figure 2, in aqueous buffer without NaCl, the rate of enzyme deactivation at 90°C is roughly the same at 7 atm and 500 atm. However, at 500 atm in 2 M NaCl the enzyme's half-life at 90°C increases by about 23-fold. The salt itself increases the half-life by a factor of 3. Thus, the combination of high pressure and high NaCl concentration has a potent stabilizing effect on the crude enzyme. The interplay between pressure and salt may also be important in the deep-sea environment.

Comparison of hydrogenases from *Mc. jannaschii* and *Mc. thermolithotrophicus*: We have also initiated studies of hydrogenase(s) from *Methanococcus thermolithotrophicus* as part of our plan to compare the effects of pressure on hydrogenases from deep-sea and shallow-water thermophiles. *M. thermolithotrophicus* is a thermophilic methanogen isolated from geothermally heated sea sediments at a depth of about 0.5 m. This organism grows between 30°C and 70°C and therefore is notably less thermophilic than *M. jannaschii*. Recently we purified

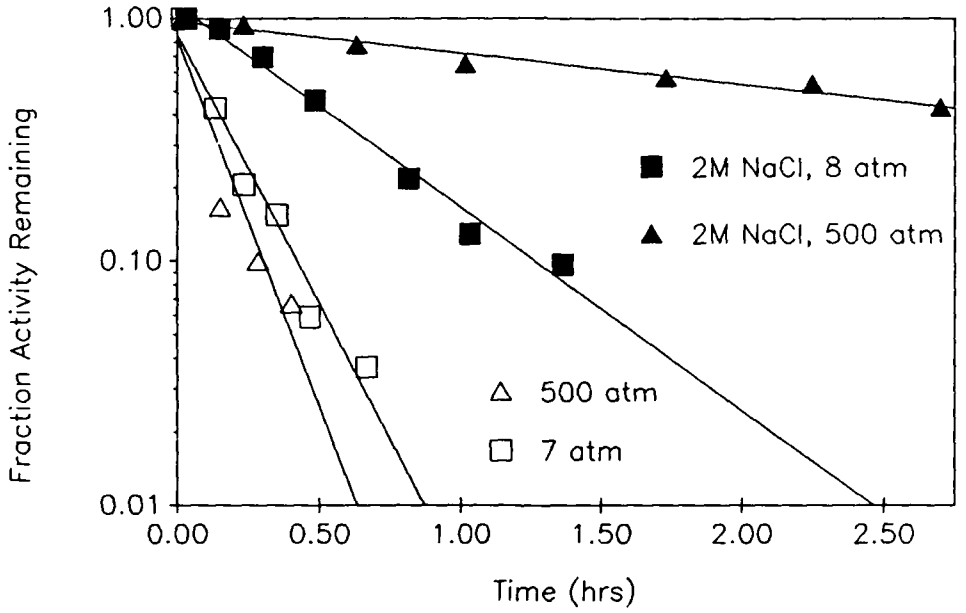

Figure 2. Remaining activity of crude hydrogenase from *M. jannaschii* versus pretreatment time at 90°C with and without salt, at high and low pressure. The substrate was methyl viologen.

an F_{420}-reactive hydrogenase from *M. thermolithotrophicus* and determined that the enzyme is similar in size and subunit composition to F_{420}-reactive hydrogenase from *M. jannaschii*. Each enzyme consists of three subunits and each forms a high molecular-weight aggregate of similar size ($M_r \approx 990$ kDa). Further characterization of the *M. thermolithotrophicus* enzyme is underway.

WORK PLAN (Year 2): Studies of bacterial growth and productivity at elevated pressures will proceed in parallel with studies of hydrogenase structure and function. The larger of our two high pressure-temperature bioreactors will be used to grow *M. janaschii* at 7.8, 250, and 500 atm. Pressure effects on cellular protein production will be examined by electrophoresis. In addition, high resolution transmission electron microscopy will be employed to investigate pressure-induced changes in the morphology of *M. jannaschii*.

In related experiments, the newly isolated strain ES4 will be grown at elevated pressures to determine if pressure increases the growth rate and/or extends the organism's upper temperature limit of growth.

To facilitate detailed structural studies of hydrogenase, we have made arrangements to grow *M. jannaschii* on a 100-L scale. Specifically, Dr. Juergen Wiegel (Department of Microbiology, University of Georgia) has agreed to carry out the fermentation. We will then purify hydrogenase, pressurize the enzyme, and send rapidly-frozen samples to Professor Michael Adams (Department of Biochemistry, University of Georgia) for low-temperature EPR analysis. These studies will determine whether hydrogenases from *M. jannaschii* contain iron-sulfur centers characteristic of other hydrogenases, and will probe the effects of pressure on any iron clusters present in the enzyme.

Pressure-induced stabilization of hydrogenases from *M. jannaschii* will be further studied at higher temperatures. Electrophoresis will be used to determine whether enzyme inactivation is caused by dissociation of the oligomeric enzyme. Analogous studies will be performed with hydrogenase from *M. thermolithotrophicus*, and possibly with a monomeric enzyme from a mesophilic organism. The ultimate goal of these experiments is to determine the extent to which pressure will stabilize enzymes under the appropriate conditions, and to elucidate the inactivation mechanism(s) inhibited by pressure.

PUBLICATIONS AND REPORTS (Year 1): Publications related to this project that acknowledge ONR support are listed below. Those marked with asterisks were submitted during the first year of the current contract.

Publications

J.F. Miller, N.N. Shah, C.M. Nelson, J.M. Ludlow, and D.S. Clark, "Pressure-Temperature Effects on the Growth and Methane Production of the Extreme Thermophile *Methanococcus jannaschii*," Appl. Environ. Microbiol., **54**, 3039 (1988).

E.L. Almond, A.J. Clark, and D.S. Clark, "Complementation of a *thr-1* Mutation of *Escherichia coli* by DNA from the Extremely Thermophilic Archaebacterium *Methanococcus jannaschii*," Appl. Microbiol. Biotechnol., **30**, 148 (1989).

J.F. Miller, C.M. Nelson, J.M. Ludlow, N.N. Shah, and D.S. Clark, "High Pressure-Temperature Bioreactor: Assays of Thermostable Hydrogenase with Fiber Optics," Biotechnol. Bioeng., **34**, 1015 (1989).

*N.N. Shah and D.S. Clark, "Partial Purification and Characterization of Two Hydrogenases from the Extreme Thermophile *Methanococcus jannaschii*," Appl. Environ. Microbiol., in press.

*D. S. Clark and R. M. Kelly, "Microorganisms at Extreme Temperatures and Pressures: Engineering Insights," Chemtech (invited paper), in press.

Presentations

C. Nelson, N. Shah, J. Ludlow, D. Hei, and D.S. Clark, "Catalytic and Structural Properties of Extremely Thermophilic Hydrogenases," AIChE Annual Meeting, San Francisco, CA, November, 1989.

D. Hei, C. Nelson, N. Shah, and D.S. Clark, "Catalytic and Structural Properties of Extremely Thermophilic Hydrogenases," poster presented at Enzyme Engineering X, Kashikojima, Japan, October 1989.

TRAINING ACTIVITIES: Two graduate students are being supported by this ONR contract:

Mr. Derek Hei

Mr. Chad Nelson